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HIV (Human immunodeficiency virus) causes a persistent infection that results in AIDS (acquired immune deficiency syndrome), a major health hazard to military and civilian populations. We have and continue to design and synthesize a variety of amino acid peptides from proteins of HIV-1 and HIV-2 viruses. Our purpose is to map the immunodominant domains of HIV-1 and HIV-2. This information and use of such reagents would accomplish three purposes. First, and under current evaluation, sensitive and specific reagents to diagnosis HIV-1 and HIV-2 infected individuals, define both viruses and mark emerging families of variants would be obtained. Second, the pathogenesis as regards the loss of, or conversely, the evolution of new immune (potentially imunopathologic) responses could be charted. Third, the data collected would be important for the design of subunit vaccines.

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Midterm Scientific Report

1. Introduction Overview

During the past contracted year we have focussed on three major issues related to the work described in contract proposal number DAMD17-88-C-8103. These are:

i. Synthesis of designated peptides designed to be reagents for detection of HIV-1 and HIV-2 infection

ii. Testing of such designed peptides to detect B cell (antibody) and T cell (proliferation) immune responses in HIV infected individuals

iii. Generate reagents (monoclonal antibodies) to use as probes to evaluate the small subset of African patients that are antibody positive to both HIV-1 and HIV-2

2. Specific Accomplishments

i. Synthesis of designated peptides designed to be reagents for detection of HIV-1 and HIV-2 infection

The following peptides have been synthesized:

a) gp41 authentic sequences or hybrid molecules of HIV-1 and HIV-2 B cell immunodominant epitopes

HIV-1

L G I W G C S G K H I C
L G M W G C S G K L I C
L G I W G C S G K L I C
L G L W G C S G K L I C
L G L W G C S G K L I S
G L W G C S G K L I C
L W G C S G K L I C
W G C S G K L I C
G C S G K L I C
C S G K L I C
S G K L I C
L W G C S G K L

HIV-2

M S W G C A F R Q V C
W G C A F R Q V C
C A F R Q V C

Hybrid HIV-1 & HIV-2

W G C A G K Q I C
W G C A F R Q V C G G G C S G K L I C
W G C A F R Q V G G G C S G K L I C
W G C A F R Q V G G G S G K L I C
W G C A F R Q V G G G K L I C

b) HIV-1 Peptides Obtained for Use in T Lymphocyte Proliferation Assay

Peptide Designation	Amino Acid Sequences	
gp41-1	603-614	
-2	609-620	
-3	655-667	
-5	737-749	
-7	584-609	(Immunodominant B cell epitope)
gp120-1	108-119	
-2	115-126	
-4	296-312	
-5	368-377	
-6	74-85	
-7	233-244	
-8	179-486	
P24-1	466-473	
-2	439-446	
-3	228-235	
-4	22-29	
-11	282-301	
pol-1	899-913	(Elects neutralizing antibody in rabbits)
-2	923-937	
-3	942-954	
-4	720-730	

c) The amino peptides (consecutive sequences) of HIV-1 P24:

P I V Q N I Q G Q M V H Q A I S P R T L N A W V K V V E E K
V H Q A I S P R T L N A W V K V V E E K A F S P E V I P M F
N A W V K V V E E K A F S P E V I P M F S A L S E G A T P Q
S A L S E G A T P Q D L N T M L N T V G G H Q A A M Q M L K
G H Q A A M Q M L K E T I N E E A A E W D R V H P V H A G P
D R V H P V H A G P I A P G Q M R E P R G S D I A G T T S T
G S D I A G T T S T L Q E Q I G W M T N N P P I P V G E I Y
N P P I P V G E I Y K R W I I L G L N K I V R M Y S P T S I

11. Testing of such designed peptides to detect B cell (antibody) and T cell (proliferation) immune responses in HIV infected individuals

a) The gp41 immunodominant B cell epitope peptides, such peptides with single amino acid substitution or HIV-1 and HIV-2 hybrid peptides were tested for their ability to bind antibodies from HIV-1 infected USA or African patients, HIV-2 infected African patients or African and USA patients without HIV-1 or HIV-2 infections (controls). Table 1 displays the results obtained. Two points can be made from the data. First, designed peptides can detect HIV-1 or HIV-2 infected individuals with sensitivity >99% and specificity approaching 100%. Hence, over 600 HIV-1 sera or over 85 HIV-2 sera tested by us, CDC (data not shown), WHO (data not shown) or the Research Laboratory of Immunology, Shanghai Institute of Biolcgical Products (data not shown), >99.9% have been

shown to be HIV specific. Further, over 400 controls tested including pregnant women or Africans without HIV infection were recorded as negative. The second point is that hybrid peptides:

W G C A G K Q I C
W G C A F R Q V C G G G C S G K I I C
W G C A F R Q V G G G C S G K L I C

pick up antibodies to both HIV-1 and HIV-2.

Table 1

	Exp 1	Patients (# positive/total) HIV-2	HIV-1 Zaire	HIV-1 USA
HIV-2	L N S W G C A F R Q V C	10/10	2/33	1/40
HIV-1 Zaire	L G M W G C S G K H I C	0/10	33/34	35/40
Zaire	L G I W G C S G K H I C	0/10	32/34	39/40
USA	L G I W G C S G K L I C	0/10	33/38	40/40
Zaire	L G L W G C S G K L I C	0/10	33/38	162/163
	Exp 2: Hybrid Peptides			
	N S W G C S G K L I C	0/4		20/20
	N S W G M W G C S G K H I C	0/4		17/20
	G M W G C A F R Q V C	3/4		0/20
	Exp 3: Hybrid Peptides		SIV*	
HIV-2	N S W G C A F R Q V C	11/11	3/3	0/10
HIV-2	W G C A F R Q V C	10/11	3/3	0/10
Hybrid 5	W G C S F R Q I C	5/11	3/3	1/18
6	W G C A G R Q I C	ND	0/3	1/8
7	W G C A F K Q I C	10/11	3/3	8/18
8	W G C A F R L I C	ND	0/3	1/8
9	W G C A F R H I C	7/11	3/3	5/18
10	W G C A G K L V C	ND	0/3	6/8
11	W G C S F K L V C	ND	0/3	1/8
12	W G C S G R L V C	ND	0/3	2/8
13	W G C S G K Q V C	ND	0/3	3/8
HIV-1	W G C S G K L I C	ND	0/3	10/10
HIV-1	L G L W G C S G K L I C	0/10	0/3	10/10

Exp 4: Hybrid Peptides

Hybrid Designed Peptides	Cont UNNF	HIV+ HIV-1 Exp 1/Exp 2	HIV-2 Exp 1/Exp 2	HIV-1 + -2
6C: W G C A G K Q I C	0/13	16/17	10/11	20/21
7A: W G C A F R Q V C G G G C S G K L I C	0/13	16/17	11/11	21/21
7B: W G C A F R Q V G G G C S G K L I C	0/13	16/18	10/11	20/21

*Because of shortage of HIV-2 sera, we used sera from simean immunodeficiency virus infected monkeys.

These peptide ELISA assays have been described (Gnann, et al. Science 237:1346-1349, 1987; Gnann, et al. Journal of Infectious Disease 156:261-267, 1987). Briefly, peptide solutions in PBS were air-dried in polyvinyl 96-well microtiter plates to yield 1 µg of peptide per well. The plates were sequentially incubated with test sera at serial two-fold dilutions, horseradish peroxidase-conjugated goat anti-human IgG, and O-phenylenediamine substrate solution, with extensive washes between steps. OD was analyzed on an automated spectrophotometer scanner at 492 nm. The cutoff for positivity was defined as the mean OD_{492} plus 3 SD for a panel of negative control sera. All sera were tested at least three times.

b) Our next studies focussed on the B cell response to immunodominant HIV-1 domain aa598-609 of HIV-1 gp41 in patients recently infected with HIV: the T cell dependency of this antibody response and the T cell proliferation response to this peptide.

B-cell response to aa598-609 of HIV-1 gp41. The appearance of IgM and IgG antibodies to the 12 amino acid peptide was measured in patients recently infected with HIV-1. Thirteen serum samples collected from four patients before and after seroconversion to HIV-1 were tested. For one patient, the approximate date of infection was known. In these samples, low titers of IgM antibody were detected early and then waned or disappeared in subsequent specimens. In contrast, the IgG antibody response was higher in titer and persisted. Analysis of these sera by two other techniques, conventional whole-virus antigen ELISA and RIPA, corroborated the results of the peptide ELISA.

Antibody response to gp41 aa598-609 is T-cell dependent. The T-cell dependence of the antibody response to peptide gp41 aa598-609 was investigated in homozygous nude (nu/nu) mice and their heterozygous (nu/+) littermates. Mice were injected intraperitoneally with peptide (100 µg) in incomplete Freund adjuvant then tested for peptide-specific IgG and IgM serum antibodies 7 and 14 days post-inoculation. The four nu/+ immunocompetent mice made high levels of IgG to the peptide at both 7 and 14 days post-inoculation. In contrast, the four T-cell deficient nu/nu mice made no IgG in response to the peptide. Both groups made low levels of specific IgM antibodies, detected on day 7 only. This absence of an IgG response to gp41 aa598-609 in the athymic mice indicates that the response is T-cell dependent, whereas the transient IgM response was T-cell independent.

T-cell proliferative response to gp41 peptide. The identification of gp41 aa598-609 as a dominant B-cell epitope led us to examine T-cell proliferation in response to this epitope. For these experiments, we used a 26 amino acid gp41 peptide (aa584-609) that included the 12 amino acid sequence. Although 100% (29 of 29) of the HIV-1 seropositive individuals tested made antibody in gp41 aa584-609, T-cells from only 7 of the 29 patients (24%) proliferated in response to this peptide, i.e., produced a stimulation index of ≥ 2 after 5 days of culture. Characteristic responses from six individuals are shown in Table 2. Donor lymphocytes were tested against gp41 aa584-609, the mitogen PHA, and HCMV antigens. Donor 1, a patient with AIDS-related complex (ARC), responded to the gp41 peptide and to HCMV and PHA. Donor 2, a patient with AIDS, responded to the gp41 peptide and PHA, but not to HCMV.

Lymphocytes from donors 3 through 6 did not proliferate to the gp41 peptide, yet the antibody titers of these donors to this epitope were equivalent to the titers observed in individuals showing proliferation. The proliferative response was mediated by T cells, as indicated by increased production of interleukin 2 (IL-2) coincident with the uptake of isotope (data not shown). Cells from all 13 HIV-1 seronegative donors tested did not proliferate in response to the HIV-1 peptide.

TABLE 2

T Lymphocyte Proliferation Response to HIV-1 gp41 Peptide[a]

Donor	Clinical Stage[b]	Antibody Added	Proliferative T cell response to: PHA	CMV	HIV-1 gp41 (aa584-609)[c]	ELISA antibody titer to HIV-1 gp41 (aa584-609)[c]
1	C	-	130.6	7.6	3.9	1:3200
		+	100.9	5.9	4.6	1:3200
2	A	-	115.9	<2	2.6	1:800
		+	102.4	<2	2.5	1:800
3	C	-	56.9	9.5	<2	1:6400
4	B	-	42.0	<2	<2	1:3200
5	B	-	168.7	8.3	<2	1:3200
6	D	-	16.5	<2	<2	1:1600

a Cells were cultured 5 days with peptide antigen or mitogen.

b C = ARC, no active disease; A = AIDS, B = ARC, with active disease, D = Asymptomatic but seropositive with immune abnormalities.

c Of the 30 HIV-1 seropositive individuals with antibodies to the gp41 peptide, only 7 responded to gp41 aa584-609 with a proliferation index >2 (range 2.1 to 7). Two of these individuals (donors 1 and 2) are shown. The other 22 failed to show a proliferative response (stimulation index <2).

The T cell proliferation assay was done as follows. Heparinized blood from HIV-1 seropositive and seronegative controls was processed on Ficoll-Hypaque gradients. The resulting lymphocyte and monocyte band was washed twice in 0.9% saline with 4 mM EDTA, and once in saline alone, then resuspended at 1 x 10^6 cells/ml in RPMI with 7% normal human plasma (type AB; seronegative for HIV-1 and human cytomegalovirus (CMV) and herpes simplex virus (HSV), 2 mM glutamine, 50 mM penicillin, and 50 mM streptomycin. Cells were plated in 96-well round-bottom microtiter plates (200 µl/well). Plates contained peptides at 100 or 50 µg/well, HSV-1 KOS (inactivated) or CMV strain AD169 at 10^5 plaque-forming units/well (T-cell recall antigens) in triplicate wells. Plates were stored at -20°C and sterilized under a UV light (1200 erg/cm^2/sec) for one hour prior to addition of cells. Cultures were incubated for 4-5 days, pulsed with 1 µci/well of ^{3}HTdR and harvested 16-24 hours later using a Cambridge Technology PhD Cell Harvester (Cambridge, MA). Filters were counted for uptake of the radioactively labeled stimuli using a liquid scintillation counter, and triplicate counts were averaged. Responses are given as a stimulation index (an index of two or more was considered significant). Control peptides were derived from murine lymphocytic choriomeningitis virus (LCMV) sequences. Cells from HIV-1 infected individuals did not respond to murine lymphocytic choriomeningitis virus peptides; cells from nine HIV-1 seronegative individuals failed to respond when incubated with HIV-1 peptides.

Proliferative response of T cells from HIV-1 infected patients to 21 HIV-1 Peptides. We next tested the responses of T lymphocytes from 29 HIV-1 infected individuals to a spectrum of peptides from the gp41, gp120, p24 and pol regions. These T cell proliferative responses were compared to B cell (antibody) responses for the same peptide. As seen in Table 3, T cells responded to peptides from several HIV-1 proteins, but IgG binding focused on the immunodominant epitope represented by peptide 41-7. Also, variation among individual patients was more evident with respect to T-cell proliferation than to antibody reactivity. Only 24% of HIV-1 seropositive donors had T-cells that proliferated in response to gp41-7, yet >99% had IgG directed to this epitope. In contrast, few individuals produced antibody that recognized any of our synthetic peptides derived from the sequence of gp120, p24, or polymerase, whereas a high percentage of HIV-1 infected subjects had T-cells that proliferated in vitro when stimulated by peptides from those proteins (e.g., gp120-2: 38%, p24-11: 24%, pol-3: 48% - Table 3).

Table 3

Comparison of T Lymphocyte Proliferation with Antibody Recognition of HIV Peptides in HIV Infected Individuals

Peptide	aa sequence	T cell prolif.[a] (SI>2) %	Antibody (IgG by Elisa) %
gp41			
1	603-614	12/29[b] (41.3)[c]	32/53 (60)
2	609-620	14/29 (42.3)	19/53 (35)
3	655-667	11/29 (37.9)	2/53 (3.8)
4	737-749	6/29 (20.7)	2/53 (3.8)
7	584-609	7/29 (24.1)	53/53 (100)
gp120			
1	108-119	9/29 (31.0)	3/53 (5.7)
2	115-126	11/29 (37.9)	2/53 (3.8)
4	296-312	4/29 (13.8)	0/53 (0)
5	368-377	6/29 (20.7)	1/53 (1.9)
6	74-85	3/29 (6.9)	0/24 (0)
7	233-244	3/29 (6.9)	1/24 (4)
8	179-486	0/29 (0)	0/24 (0)
p24			
1	466-473	4/29 (13.8)	2/53 (3.8)
2	439-446	4/29 (13.8)	2/53 (3.8)
3	228-235	4/29 (13.8)	4/53 (7.5)
4	22-29	4/29 (13.8)	4/53 (7.5)
11	282-301	7/29 (24.1)	0/53 (0)
pol			
1	899-913	8/29 (27.6)	2/53 (3.8)
2	923-137	11/29 (37.9)	1/53 (1.9)
3	942-954	14/29 (48.3)	2/53 (3.8)
4	720-730	10/29 (34.5)	3/53 (5.7)

a. A positive response is defined as a stimulation index of 2 or more after 5 days of culture.
b. Denominator is total number of HIV seropositive tested.
c. Percentage of those responding.

The correlation of some HIV-1 patients' clinical parameters with peptide responses is observed in Table 4. Of the HIV-1 seropositive individuals tested 90% responded to at least one (and up to 14) of 21 peptides. However, cells from nine HIV-1 seronegative controls did not respond (stimulation index less then 2) to these HIV-1 peptides but did respond appropriately to PHA, CMV, and HSV (T cell recall Ag) (data not shown). The most striking observation in the HIV-1 seropositive group was the variability in individual peptide response patterns. In fact, no single peptide activated cells from all HIV-1 infected donors. However, peptides from particular proteins consistently generated the most responses, e.g., peptides derived from gp41 stimulated twice as many responses as gag peptides.

Lack of immune response may be caused by virus-induced immunodeficiency. Indeed, decreasing immune capability with worsening disease was borne out by the number of times lymphocytes responded to different test peptides: for AIDS patients 2.0, for ARC 3.3, for asymptomatic ARC 5.3, and for healthy HIV-1 seropositive patients 8.1 peptides. The best T cell responses were from the healthiest patients. The magnitude of the response also varied according to peptide (e.g., gp41-1 X SI=3.0; gp120-2 x SI=4.7) although SI were generally low. Some peptides were recognized by more individuals than were other peptides. For instance, cells from 14 of 29 (48%) individuals proliferated in response to gp41-2 or pol-3 but 0/29 responded to gp120-8. The most reasonable explanation for the variability in T cell response is the pleomorphism of the major histocompatibility complex glycoproteins (MHC) and the fact, as shown in mice, different MHC glycoproteins select different peptides.

Table 4

Correlation of a Number of Responses to HIV Peptides and CMV with CKT4+ T Cell Numbers and Delayed Hypersensitivity in HIV Infected Individuals

Patient	Stage[a]	T Cell Proliferation (SI>2) Peptides							
		gp41(5)[b]	gp120(7)[c]	pol(4)[d]	gag(5)[e]	Total	CMV[f]	CD4 number (per cm³)	Skin Reactivity (out of 5)
1291	A	0	0	0	0	0	-	ND	ND
250	A	1	1	2	0	4	-	36	1
479	B	1	0	0	1	2	+	83	0
1300	B	1	0	3	1	5	+	ND	ND
'99	B	0	1	1	4	6	-	62	2
839	B	2	1	1	1	7	-	171	0
297	C	1	1	0	0	2	+	95	1
209	C	1	1	2	0	4	-	313	1
463	C	2	2	1	2	1	+	532	0
242	C	2	2	2	1	7	+	580	3
603	C	3	5	4	0	12	+	797	2
413	C	0	6	0	0	6	+	470	1
414	C	3	1	3	1	8	-	873	1
351	C	2	1	1	0	4	+	799	0
291	C	2	0	3	4	9	+	966	2
336	C	4	3	2	0	9	+	791	1
383	C	4	3	2	0	9	+	882	2
341	C	0	0	0	0	0	-	526	2
341	C	2	0	1	0	3	+	766	1
262	C	2	1	2	1	6	-	436	0
231	C	1	0	0	0	1	-	530	2
326	C	0	1	2	1	4	-	123	3
388	D	3	2	3	1	9	+	482	0
224	D	1	0	0	0	1	+	712	2
1319	D	5	6	3	0	14	+	ND	ND
395	D	2	1	1	2	5	-	51	0
423	D	3	0	4	4	11	+	1167	2

a. A = AIDS, B = ARC, C = Asymptomatic ARC, D = HIV Ab. positive with immune abnormalities
b. Number of responses out of 5 gp41 peptides
c. Number of responses out of 7 gp120 peptides
d. Number of responses out of 4 pol peptides
e. Number of responses out of 5 gag peptides
f. T-cell proliferation to CMV (all donors were seropositive).

iii. Generate reagents (monoclonal antibodies) to use as probes to evaluate the small subset of African patients that are antibody positive to both HIV-1 and HIV-2. Recently, serologic studies of HIV+ west African individuals indicated that a significant number (varying 3 to 14%) possessed antibodies to both HIV-1 and HIV-2 in their sera despite the use of HIV specific peptides and other segregating procedures. These observations suggest three possibilities, none of which need exclude the other. This first is that a single individual is infected with both HIV-1 and HIV-2. Evidence to support this contention has recently come forth from two individual patients when using specific sequences for HIV-1, for HIV-2 and the polymerase chain reaction protocol to amplify DNA in peripheral blood mononuclear cells (PBMC) or by direct isolation of both viruses from PBMC. However, experience of many laboratories has been the inability to confirm dual infection of the majority of individuals carrying antibodies to both HIV-1 and HIV-2. This suggests that dual infection may be an uncommon event. A second possibility is the formation of a single recombinant virus, an event previously observed with some but not all persistent murine retrovirus infection. As yet there is no evidence for a noval recombinant virus in AIDS. The third possibility is immunologic cross-reactivity between amino acid linear determinant(s) or conformational shape shared between HIV-1 and HIV-2.

To address this last possibility we generated monoclonal antibodies to HIV-1 gp41 immunodominant domain LGLWGCSGHIC and the minimal recognition epitope CSGKLIC. Two monoclonal antibodies that recognize CSGKLIC of HIV-1 also cross-react with the immunodominant gp41 epitope of HIV-2 amino acid sequence CAFRQVC. Hence, cross-reaction likely occurs to confirmation determinants of HIV-1 and HIV-2.

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